(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00041 A2

(51) International Patent Classification[7]: A23J 3/00, 3/28, 3/16, A61K 9/16

(21) International Application Number: PCT/NL00/00451

(22) International Filing Date: 28 June 2000 (28.06.2000)

(25) Filing Language: Dutch

(26) Publication Language: English

(30) Priority Data:
1012452 28 June 1999 (28.06.1999) NL

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/00041 A2

(54) Title: METHOD OF PREPARING PROTEIN AGGLOMERATES, PROTEIN AGGLOMERATES, AND A FOOD PRODUCT AND A PHARMACEUTICAL COMPOSITION IN WHICH THEY ARE COMPRISED

(57) Abstract: The invention relates to a method for the preparation of protein agglomerates, by introducing CO_2 in an aqueous protein-containing solution. According to the invention CO_2 is gradually and while mixing supplied yielding spherical protein agglomerates, after which the pressure is reduced with a rate such that the spherical nature of the protein agglomerates is substantially maintained. The invention also relates to a food product and a pharmaceutical compound containing such protein preparations.

Method of preparing protein agglomerates, protein agglomerates, and a food product and a pharmaceutical composition in which they are comprised

The present invention relates to a method for the preparation of protein agglomerates, wherein in an aqueous protein-containing solution having a pH above the iso-electric point of the protein from which agglomerates are to be formed, carbon dioxide is dissolved at elevated pressure, causing the pH of the protein-containing solution to fall until the pH of the solution substantially reaches the iso-electric point of said protein.

Such a method is known in the art. Jordan P.J. et al. (J. of Dairy Science and Technology, 22, pp. 247 (1987)) disclose a method for the preparation of a crude casein-agglomerate using carbon dioxide at elevated pressure (3500 kPa (35 Bar)). The agglomerate had the appearance of curd and the protein yield was 99%.

The object of the present invention is to provide a method for the preparation of high-grade protein agglomerates.

To this end, the method is characterized in that CO_2 is introduced gradually and under mixing yielding spherical protein agglomerates, after which the pressure is reduced at a limited rate in order to substantially preserve the spherical nature of the protein agglomerates.

Surprisingly it has been found that proteins exist which may be formed into spherical agglomerates by controlled agglomeration. Such spherical protein agglomerates may be applied in various fields. Whether agglomerates can be formed from a protein depends on a number of factors, which include the solubility of the protein in the solvent and the charge distribution. A person skilled in the art can experimentally examine the suitability without undue effort and in a simple manner. Although some

form of mixing is thought to be required, care should be taken to avoid the formation of foam and forces that may destruct spheres that are formed. When mention is made of substantially reaching the iso-electric point (IEP), this is understood to be a pH equal to the IEP ± 1, but preferably ± 0.6 or less. The deviation allowed depends on the type of protein and the concentration of protein, both of the protein itself and of other proteins present that have a comparable IEP. At higher protein concentrations a larger deviation in pH is allowed. CO_2 is gradually introduced into the solution (limited by the surface area of the liquid-gas interface and the rate of mixing), even if the liquid is immediately put under a high CO_2 pressure. The rate of mixing is to be limited in such a way that spherical protein agglomerates are not destroyed by shear. The protein content of a spherical protein agglomerate according to the present invention is very high, in general >80% based on the dry weight of an agglomerate.

Animal proteins or proteins formed by fermentation processes, such as produced by bacteria, may be used for the protein-containing solution. Suitably, a vegetable protein-containing solution is used as the protein-containing solution.

Thus it is possible to apply vegetable proteins for a higher grade application. In this respect protein agglomerates in food products may be considered, wherein the spherical nature may contribute to realize improved organoleptic properties.

The vegetable proteins may, for example, be protein derived from wheat, corn, sunflowerseeds, and coconuts, but according to a preferred embodiment the vegetable protein is soya protein.

Soya protein is readily and cheaply available but does not always have the properties desired for food technological applications. Using the method according to the present invention, the two most important proteins can be separated, wherein the protein having the highest iso-

electric point also has the highest sulphur content. As one of the disadvantages of soya protein is the low sulphur content, a soya protein fraction enriched in sulphur-containing amino-acids according to the present invention may find application on a larger scale. Using the method according to the present invention, spherical (soya)protein agglomerates can be formed having a diameter in the range of 2 to 50 μm, with a limited variation in particle size.

According to a preferred embodiment the pH is kept above 5.3.

Thus it is avoided that phytate ends up in the protein agglomerates.

For the production of protein agglomerates having a small diameter, it is preferred that the protein concentration in the protein-containing solution be less than 0.5 g/l.

According to an interesting embodiment, the protein-containing solution contains more than 1 protein, from which proteins protein agglomerates are formed by lowering the pH.

By acidifying the solution using CO_2 strongly and (at least relatively) quickly, spherical protein agglomerates can be formed which are a mixture of the proteins. By introducing CO_2 step-wise, spherical protein agglomerates can be formed wherein each protein agglomerate is comprised substantially of one protein.

According to an interesting embodiment therefore, protein agglomerates formed from a protein are separated before (at a higher CO_2 pressure) a further protein is formed into protein agglomerates.

Thus separated fractions of protein agglomerates can be obtained.

When the protein agglomerates are not removed it is possible to coat them with the second protein. It is also possible to form very large spherical protein agglomerates by using earlier formed protein agglomerates as nucleating material.

The invention encompasses stripping a carbon dioxide-containing protein-containing solution to give a solution depleted in carbon dioxide wherein, as a result of stripping, spherical protein agglomerates are formed.

This method is suitable for proteins having an iso-electric point around 7 (such as 6 - 8). Thus a protein may be extracted using a solution which is prepared by introducing carbon dioxide into a slightly basic solution (suitably of a base having a pK_b <4). Stripping may be achieved using any method known in the art, such as contacting with a gas low in carbon dioxide, such as nitrogen, which nitrogen enriched in carbon dioxide is discharged.

According to a preferred embodiment the spherical protein agglomerates formed are stabilized using an agent chosen form the group consisting of i) an acid; and ii) a cross-linking agent.

The spherical protein agglomerates may be stabilized by means of drying or, in accordance with the above embodiment, with the aid of an acid. The acid may be an organic or inorganic acid as desired, and for most applications a physiologically acceptable acid. Suitable acids are, for example, acetic acid or hydrochloric acid. Suitably, the acids are gradually introduced while carbon dioxide is discharged, keeping the pH substantially constant. The pH is allowed to rise somewhat, such as for example by up to 1 pH unit. In general, smaller increases improve the stability. Use may also be made of cross-linking agents, such as for example glutaric dialdehyde or formaldehyde. The cross-linking agents will also preferably be physiologically acceptable.

The invention more generally relates to spherical protein agglomerates, such as spherical soya protein agglomerates, prepared using the method according to the invention.

Two important possible applications of the spherical protein agglomerates according to the invention are protein-containing food products and pharmaceutical

compositions, including the preparation thereof.

Finally the present invention relates to the preparation of protein agglomerates using an apparatus comprising a first container having a first inlet and a first outlet positioned opposite to said first inlet, the first outlet being connected to a second inlet of a second container, which second container further possesses a second outlet positioned opposite to said second inlet, wherein the first container is provided with a first gas inlet for a gas rich in carbon dioxide and a first gas outlet for gas depleted in carbon dioxide positioned opposite to said first gas inlet, and the second container is provided with a second gas inlet for gas low in carbon dioxide and a second gas outlet for a gas enriched in carbon dioxide positioned opposite to the second gas inlet, wherein the (gas)inlets and (gas)outlets are placed such that during operation fluid introduced via an inlet is in countercurrent with gas introduced via a gas inlet.

Such an apparatus allows the method to be performed continuously. To this end, a pump will generally be provided for supplying the protein-containing liquid at an elevated pressure to the first inlet of the first container, and at the same pressure gas rich in carbon dioxide will be supplied via the first gas inlet. Suitably the first container is not completely filled with protein-containing liquid and the gas rich in carbon dioxide is introduced in countercurrent above the liquid. Because of the countercurrent operation the gas rich in carbon dioxide is depleted in carbon dioxide, and a rapid decrease in pH of liquid newly introduced into the container is avoided. Similarly, in the second container gas poor in carbon dioxide, which includes gas devoid of carbon dioxide, is introduced. This gas, for example nitrogen or air, takes up carbon dioxide from the liquid introduced via the second inlet, which gas enriched in carbon dioxide is discharged via the second outlet. If desired, this gas may be purified or supplemented with CO_2 and fed to the first gas inlet of the first container.

Preferably the containers are elongated.

This provides a simple method of a gradual change in pH, and limits the effect of mixing in the liquid's direction of flow.

Mixing occurs in a direction substantially perpendicular to the liquid's direction of flow.

Such a mixing can readily be achieved by stirring means rotating perpendicular to the direction of flow. This promotes mixing in the radial direction without much mixing occurring in the axial direction.

The present invention will now be illustrated with reference to the following non-limiting example and with reference to the drawing in which

Fig. 1 is an electron micrograph of protein agglomerates according to the invention; and

Fig. 2 is an electron microscopic enlargement of a protein agglomerate.

Example I

1 Part by weight of de-fatted soya flour is mixed with 9 parts by weight demineralized water. The mixture is stirred for 30 minutes at 25°C, and the pH is maintained at 9 using 1 M NaOH. Subsequently the mixture is centrifuged for 2 hours at 4000 g. The almost clear supernatant is a protein-containing stock solution (protein concentration circa 40 g/l) which is used in a dilution (with water) of 1:200, 1:9 and 1:1 for the preparation of protein agglomerates. 500 ml diluted protein-containing solution is transferred to a pressure vessel (volume 1 l.). To acidify the diluted protein-containing solution, CO_2 is introduced at 25°C above the liquid. Stirring (tilted blade stirrer; diam. 4.6 cm) occurs such that the formation of foam is substantially avoided. CO_2 is introduced until the pH=4.8, as measured using a high pressure pH sensor (Büchiglas, Uster, Switzerland). After maintaining this pressure for at least 30 seconds, the pressure is gradually reduced (in respectively 5, 25 and 60 sec.). A milky white suspension is obtained, which is diluted 1:100 with 0.07 M sodium acetate pH 4.8. The particle size is deter-

mined using a Coulter counter and is, based on volume, 7 μm, 25 μm and 35 μm respectively. As visible in Fig. 1, the width of the size distribution of the spheres formed using the method is limited. In Fig. 2 it can be seen that a sphere formed during the method according to the invention is porous. Both properties provide special organoleptic properties. Furthermore the defined spheres are, as a result of this, suitable for the reliable (reproduceable) preparation of food products. The same goes for pharmaceutical compositions in which the spheres may be used as an excipient or carrier. Of course, if the active ingredient is a suitable protein, it may be formed into a spherical protein agglomerate.

Example II

The method of Example I was repeated with a 1:1 dilution of the soya flour extract. The difference with the experiment of Example I was that the rotational speed was varied (see table below).

Rotational speed (rotations per minute)	Power input (milliWatt/kg soya protein solution)	Product of rate of acidification and shear rate (no dimension)	Particle size (μm)
50	0.2	19000	31.4
300	50	13000	38.3
800	980	45000	27.0

In all instances analysis using electron microscopy showed spherical particles having the mean particle size indicated in the table.

Example III

Example I was repeated (using 750 ml 1:1 diluted soya flour extract) at 300 rpm. Immediately after the desired pH was attained, the rotational speed was reduced to 50 rpm. Carbon dioxide in the reactor vessel was eliminated while maintaining the pressure by supplying nitrogen. Nitrogen was supplied for 5 minutes at a rate of 0.5 litre per minute. By operating this way reduced formation of foam was observed when the pressure was reduced.

Example IV

The experiment of Example I was repeated (using 750 ml 1:1 diluted soya flour extract. Rotational speed 300 rpm; after reaching the desired pH reduced to 50 rpm) but before the pressure was reduced, 10 ml 25% glutaric dialdehyde solution was added. Thus stabilized spherical protein agglomerates were obtained which proved to be sterile over a large pH range, even after storage for several weeks.

Example V

The experiment of Example I was repeated with 500 ml undiluted stock solution, and during the reduction of the pressure 0.5 N hydrochloric acid was supplied such that the pH remained constant (pH = 4.8). The pH was kept

constant by controlling the rate at which CO_2 was discharged while acid was supplied at a constant rate of 8 ml/min. By keeping the pH slightly above the pH set originally for protein agglomeration using CO_2, further agglomeration was avoided.

The table below shows the course of pressure and pH.

Time (min.)	Pressure	pH
0	48.4	4.82
1	21.4	4.92
2	13.4	4.96
3	8.1	4.95
4	4.7	4.81
5	1.2	4.85

Example VI

Soya protein which can be precipitated using acid comprises two main components, i.e. glycinine and β-conglycinine. The respective iso-electric points are 5.2 and 4.9. Separation took place by supplying CO_2 up to a total pressure of 1.2 Bar (pH=6), after which the solution was removed from the reactor vessel and centrifuged using a precipitate rich in glycinine (90% pure). Subsequently, the pressure was elevated to 5.0 Bar (pH=5.2) and in the same way a precipitate was removed containing a mixture of both acid-precipitable soya proteins. Finally the pressure was raised to 30.6 Bar (pH=4.8), as a result of which a 80% pure β-conglycinine fraction was obtained.

CLAIMS

1. 1. Method for the preparation of protein agglomerates, wherein in an aqueous protein-containing solution having a pH above the iso-electric point of the protein from which agglomerates are to be formed, carbon dioxide is dissolved at elevated pressure, causing the pH of the protein-containing solution to fall until the pH of the solution substantially reaches the iso-electric point of said protein, **characterized**, in that that CO_2 is introduced gradually and under mixing yielding spherical protein agglomerates, after which the pressure is reduced at a limited rate in order to substantially preserve the spherical nature of the protein agglomerates.

2. Method according to claim 1, **characterized**, in that the protein-containing solution used is a vegetable protein-containing solution.

3. Method according to claim 2, **characterized**, in that the vegetable protein is soya protein.

4. Method according to claim 2 or 3, **characterized**, in that the pH is kept above 5.3.

5. Method according to any of the preceding claims, **characterized**, in that the protein concentration in the protein-containing solution is less than 0.5 g/l.

6. Method according to any of the preceding claims, **characterized**, in that the protein-containing solution contains more than 1 protein, which proteins are formed to protein agglomerates by lowering the pH.

7. Method according to claim 6, **characterized**, in that after forming protein agglomerates from a protein, the protein agglomerates formed are separated before a further protein is formed to protein agglomerates.

8. Method according to any of the preceding claims, **characterized**, in that the protein agglomerates are stabilized using an agent chosen from the group consisting of i) an acid; and ii) a cross-linking agent.

9. Method according to any of the preceding

claims, wherein for the preparation of protein agglomerates using an apparatus comprising a first container having a first inlet and a first outlet positioned opposite to said first inlet, the first outlet being connected to a second inlet of a second container, which second container further possesses a second outlet positioned opposite to said second inlet, wherein the first container is provided with a first gas inlet for a gas rich in carbon dioxide and a first gas outlet for gas depleted in carbon dioxide positioned opposite to said first gas inlet, and the second container is provided with a second gas inlet for gas poor in carbon dioxide and a second gas outlet for a gas enriched in carbon dioxide positioned opposite to the second gas inlet, wherein the (gas)inlets and (gas)outlets are placed such that during operation fluid introduced via an inlet is in countercurrent with gas introduced via a gas inlet.

10. Method according to claim 9, **characterized**, in that elongated containers are used.

11. Method according to claim 9 or 10, **characterized**, in that mixing occurs in a direction substantially perpendicular to the direction of movement of the liquid.

12. Spherical protein agglomerates prepared according to any of the claims 1 to 11.

13. Spherical protein agglomerates, **characterized**, in that the spherical soya protein agglomerates are prepared according to any of the claims 3 to 11.

14. Proteinaceous food product, **characterized**, in that it contains spherical protein agglomerates according to claim 12 or 13.

15. Pharmaceutical composition comprising a pharmaceutically active compound together with spherical protein agglomerates according to claim 12 or 13.

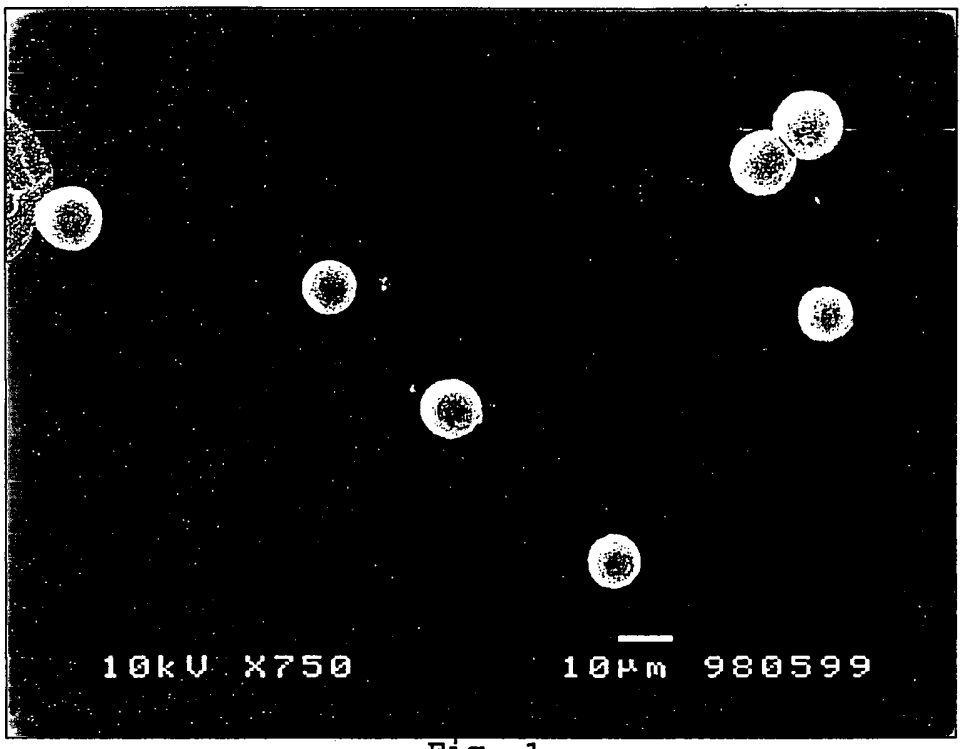

Fig. 1

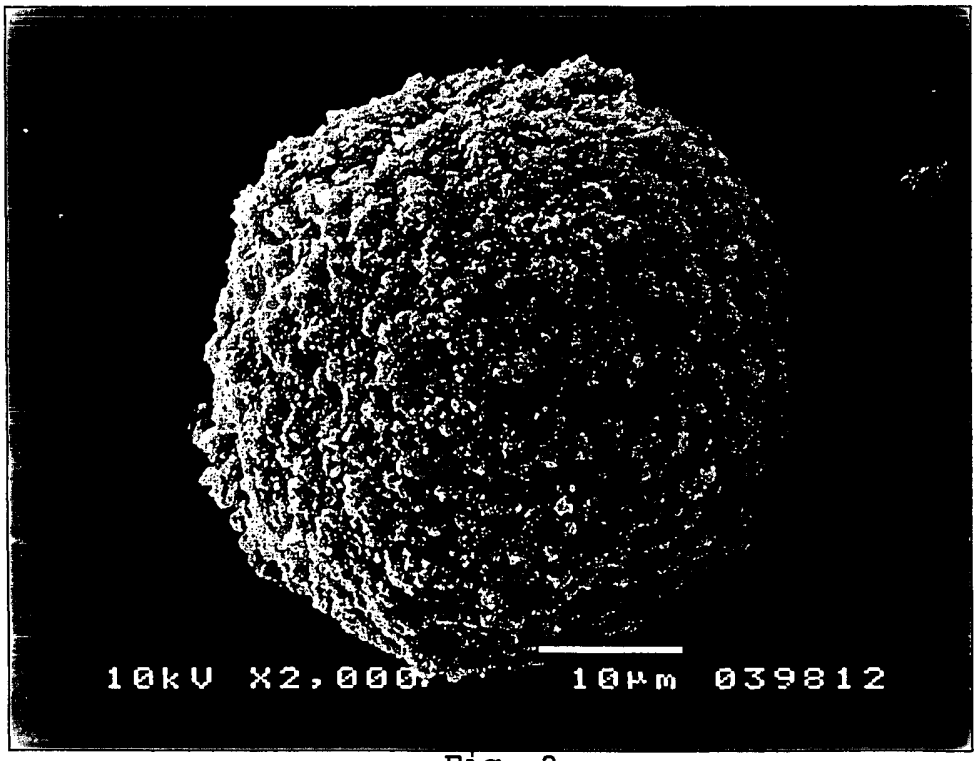

Fig. 2